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Research Article

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Transdermal and Moisturizing Effects of Novel Supramolecular Hyaluronic Acid

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Abstract

Hyaluronic acid (HA) is an important macromolecular polymer widely existing in skin, which can lock water molecules in the skin.Due to the barrier effect of skin stratum corneum, high molecular weight HA cannot enter the deep skin, while low molecular weight HA has ability limited to maintian water.Nowadays, HA injection is widely used as a supplement method, which brings many disadvantages due to its invasiveness.Therefore, in this study, the ionic liquid induction technology was used to transform the macromolecular HA into the supermolecular state. Through in vitro transdermal experiment and human skin moisturizing experiment, it was proved that the supermolecular state HA has better transdermal and skin moisturizing ability, which provide a new potential method for non-invasive exogenous supplementation of HA.

Keywords: Hyaluronic Acid, Supramolecular Technique, Transdermal Absorption, Moisturizing Effect

1. Introduction

Skin is the largest organ of the human body, and its basic structure can be divided into epidermis, dermis and subcutaneous tissue and epidermis is the outermost layer of skin.According to the differentiation and characteristics of keratinocytes, the epidermis is divided into 5 layers from outside to inside, namely stratum corneum, stratum lucidum (only exists in palm and sole), stratum granulosum, stratum spinosum and stratum basale, the basal layer is connected with dermis by means of basement membrane.

The skin provides a physical and physiological barrier to the human body, this barrier function mainly depends on the stratum corneum.The keratinocytes that make up the stratum corneum which filled with bundles of densely aggregated keratin, and play an important role of barrier [1]. It is because of this barrier effect, isolated a certain size of the material into the human body through the skin. Studies have shown that only substances with a molecular weight (MW) of less than 500Da, as well as lipophilic compounds, are generally able to penetrate the skin barrier [2].

The chemical name of hyaluronic acid is (1,4)-O-β D-glucuronic acid-(1,3)-2-acetamido-2-deoxy-β-D-glucose.It is a high-molecular linear glycan, a polymer formed by alternating N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) disaccharide units repeatedly (Figure 1), with molecular formula $(C_{14}H_{20}NO_{11}Na)_n$, and the MW of disaccharide units is 401.3 [3]. The MW of HA molecules with different lengths of polymeric linear chains varies widely, ranging from 600Da to 1,000 kDa [4].

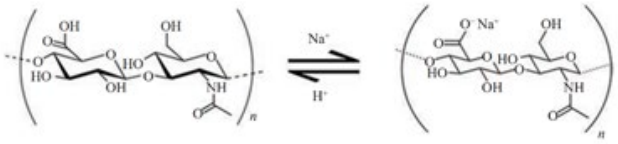

Figure 1: Structural formula of hyaluronic acid

HA is widely involved in various physiological activities of the body, such as tissue homeostasis, cell proliferation, cell migration, cell differentiation, angiogenesis, tumor activity, and anti-apoptosis [5-10]. Since Meyer and Palmer initially isolated HA from bovine vitreous in 1934, HA has been widely used in medical cosmetology, biomaterials, drug delivery, and preven-

tion of adhesion after abdominal surgery [11].

Although HA is widely distributed in the human body, the most concentrated organ of HA in our body is still the skin, which is the endogenous polysaccharide with the highest concentration in skin and connective tissue and an important component of dermal extracellular matrix (ECM) [12]. Studies have shown that the content of HA in the skin accounts for more than 50% of the total HA in the body.HA has strong hydrophilicity, and HA in aqueous solution can combine water molecules with more than 1000 times of its own mass;In addition, HA aqueous solutions are non-Newtonian fluids with good viscoelasticity and strainability [13]. Therefore,HA contributes a lot in the morphological support of the skin, moisturing, and 111maintaining extensibility and elasticity [14].

With the age increasing and ultraviolet radiating, the HA content in the skin will continue to decrease, and then there will be relaxation, wrinkles and other outcomes, resulting in a decline in skin barrier function [15,16]. Therefore, exogenous HA supplementation is of great significance in skin moisturizing and wrinkle removal and facial anti-aging in medical cosmetology.

Due to the skin barrier effect, the high molecular weight (HMW) HA can only stay on the skin surface and cannot penetrate deep into the skin.Therefore, HA micro-needle injecting has become one of the main approach of exogenous HA supplementation nowadays.However, many shortcomings attribute its invasive characteristics.How to supply HA into the deep skin non-invasively has become a very meaningful research direction.

The creation of supramolecular chemistry is a major breakthrough in the field of biology and chemistry in the last century, providing many valuable methods and tools for materials chemistry, physiology, medicine and so on.At least six scientists have won Nobel Prizes for their work in supramolecular chemistry. Supramolecular chemistry is based on the existence of molecular assemblies and intermolecular forces, and different types of molecules can interact and self-assemble according to their different strengths, orientations, and dependence on distance and angle [17]. According to the principle of hypermolecular self-assembly, the interaction force between molecules can be used as a tool to assemble components or modules with specific structures and functions into new supramolecular compounds in a certain way [18].These new compounds not only exhibit unique properties that individual molecules do not possess, but also greatly increase the variety and number of compounds [19].

In order to make HA break through the limitation of skin barrier, this study will use supramolecular technology to prepare HA molecules with different MWs into supramolecular state in specific ionic environment, and compare their transdermal effects respectively with non-supramolecular HA molecules, and evaluate their respective skin moisturing effects on human skin. (Figure 2).

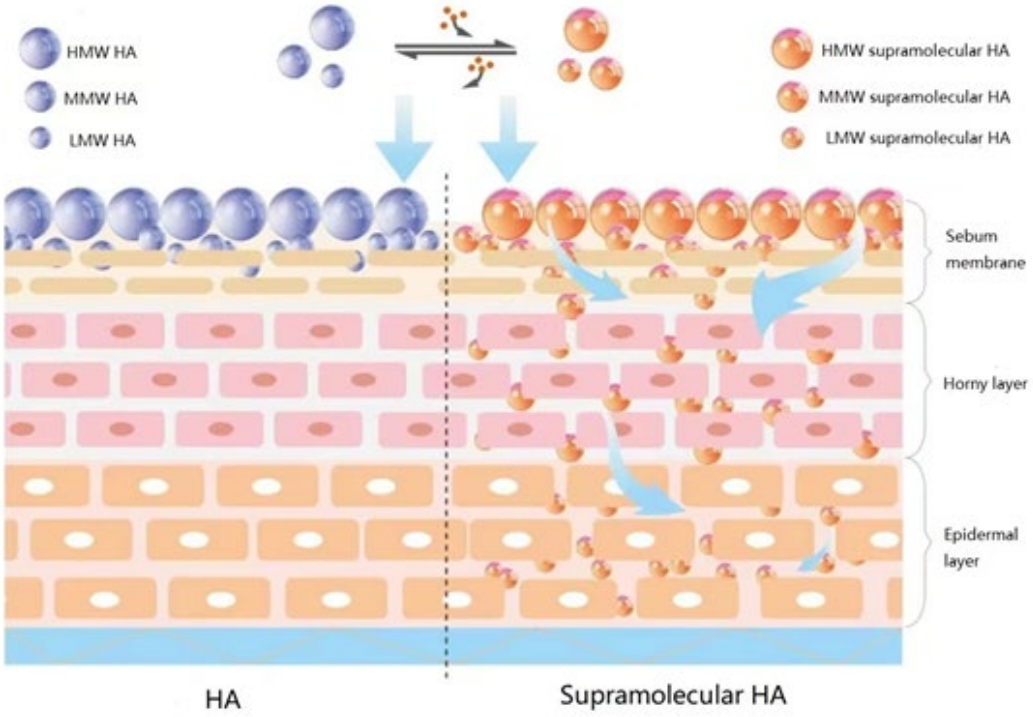

Figure 2: Schematic diagram of supramolecular hyaluronic acid transdermal principle

2. Materials and Methods

2.1 Experimental Materials

HA powders of different MW(MW 10-100kDa, 100-1,800 kDa, >1,800 kDa, 95.7%) were purchased from Huaxi Biotechnology Co., Ltd. (Beijing, China);Citric acid, potassium hydroxide, glycerol, panthenol, 1,3-propanediol, phenoxyethanol, 1,2-hexanediol, ascorbyl glucoside, p-hydroxyacetophenone, lipoic acid, carnosine, arginine, triethanolamine, DMSO, fluorescein

amine were purchased from Sigma (St. Louis, MO, USA).

2.2 HA Molecular Fluorescence Labeling Experiment

Weigh a certain amount of HA powder with different MW and dissolve in ultrapure water respectively, adjust pH to about 4.5 with concentrated hydrochloric acid, weigh a certain amount of EDC and NHS, dissolve in DMSO, add them into HA solution after full dissolution, activate for 1h at room temperature, adjust pH to 7-8 with NaOH solution;After a certain amount of fluorescein amine was dissolved in DMSO solution, it was added into the activated HA solution sample to make the ratio of-COOH to-NH2 in the solution be 1:1, and the reaction was carried out for 24h at room temperature in the dark;Add 4 times volume of absolute ethanol to the labeled HA solution, react for 5min, centrifuge at 3500g for 10min, remove the supernatant, wash the precipitate twice with 3ml absolute ethanol, centrifuge at 3500g for 3min each time, dissolve the precipitate with ultrapure water, and store at room temperature in the dark.

2.3 Preparation of Supramolecular Hyaluronic Acid

Adding a certain amount of citric acid into a potassium hydroxide solution to form a citric acid buffer solution system, adding a certain amount of glycerol, panthenol, 1,3-propanediol, phenoxyethanol, 1,2-hexanediol, ascorbic acid glucoside, p-hydroxyacetophenone, lipoic acid, carnosine, arginine and triethanolamine into the citric acid buffer solution, shaking and dissolving to obtain a supramolecular ion induction system;The fluorescence-dyed HA solution and that supramolecular ion induce system are mixed in equal volume and store at room temperature in a dark place.

2.4 Laboratory Animals

The abdominal skin of Bama miniature pig was cut immediately after being killed by anesthesia, the subcutaneous fat and connective tissue were carefully peeled off, washed with normal saline (NS) and placed in NS, and stored in low temperature refrigerator for later use.Thaw naturally before the test, soak in NS for 30 min, and blot dry with filter paper for later use. The procedures related to the laboratory animals involved in this study were in compliance with the relevant provisions in the Guidelines for Ethical Review of Laboratory Animal Welfare issued by the General Administration of Pattern Supervision, Inspection and Quarantine of China and the Standardization Administration of China.

2.5 Transdermal Penetration of Supramolecular HA in Vitro

Firstly, the skin (pig skin) was fixed between the supply chamber and the receiving chamber of Franz diffusion cell, the stratum corneum of the skin faced the supply chamber, and the dermis side faced the receiving chamber;adding a receiving liquid into the receiving chamber, tightening the piglet skin and fixing the piglet skin with the receiving pool, adding a certain volume of receiving liquid (PBS) into the receiving chamber, exhausting air, and making the skin dermis layer closely contact with the receiving liquid;supermolecular HA sample and non-supermolecular HA samples with different MW are respectively added to that skin surface in the supply chamber:adding a certain amount of HA sample to be tested to the skin surface of an independent donor, and uniformly spreading the sample from the central part of the skin to the edge in a radial manner by using a disposable gun head;Start the electromagnetic stirrer to stir at the speed of 300rpm, keep the water bath at constant temperature of (32±1) ℃, and ensure that there is no bubble in the interlayer of the water bath, and make transdermal reaction for 2 h;After the tissues were washed with water, they were fixed with formalin solution overnight, and then dehydrated in different concentrations of ethanol, transparent, waxed, embedded and sectioned. The fluorescence intensity was observed under a fluorescence microscope.

2.6 Volunteers Recruitment of Human Skin Moisturizing Experiment

The enrollment conditions of the recruited volunteers are in accordance with the number of volunteers and the condition standard for “Determination of moisture content of stratum corneum by capacitance method” in the Guidelines for Evaluation of Moisturizing Efficacy of Cosmetics (QB/T 4256-2011) issued by the Ministry of Industry and Information Technology of China, specifically, the number of volunteers is 24-30, and the enrollment age is between 18 and 65 years old;The basic value of capacitance skin moisture tester in forearm test area is between 15 and 45;No use of antihistamines in the past week or immunosuppressants in the past month;Patients who have not applied any anti-inflammatory drugs to the test site within the past two months;Non-insulin-dependent diabetes mellitus;Non-lactating or pregnant women; those who have not participated in other clinical trials at the test site at present or in the past three months;There is no scar, nevus or other factors that may affect the determination of the test results on the skin of the forearm to be tested;Voluntary participation in this trial and ability to complete the specified contents as required by the trial.

2.7 Moisturizing Experiment of Supramolecular HA on Human Skin

The test is conducted according to the test environment conditions, instruments and test procedures for “Determination of moisture content in stratum corneum by capacitance method” in the Technical Specification for Safety of Cosmetics (2015 Edition) issued by the former China Food and Drug Administration and the Guidelines for Evaluation of Moisturizing Efficacy of Cosmetics (QB/T 4256-2011) issued by the Ministry of Industry and Information Technology of China.in particular to that method for carry out real-time dynamic monitoring unde the test environment temperature of 20 DEG C to 22 DEG C and the humidity of 40 percent to 60 percent.Volunteers should not use any cosmetics or topical drugs 2 - 3 days before the test, and should not touch water 1 -3 hours before the test.Before the test, volunteers wiped and cleaned the inner forearms of both hands with dry tissue;selecting three test areas with an area of 3cm * 3cm from the inner side of the forearms of both hands of the volunteer, wherein the test areas have the same area and the interval between each area is at least 1cm, and the test areas are divided into a blank control group, a supramolecular HA group and a non-supramolecular HA group and marked;After marking, the volunteers sat quietly in the room for 30 minutes, during which time they could not drink water or beverages, and kept their forearms exposed, placed in the test state and kept relaxed.

Before the sample application, the skin capacitance value of the volunteers in the test area was measured by Corneometer® CM 825 skin moisture meter (Courage & Khazaka, Germany), and then the supramolecular HA solution, non-supramolecular HA solution and ultrapure water were evenly applied to the test area on the inner forearm respectively. The skin capacitance value of the test area was measured 4h and 8 h after the sample application.Each test area is measured in parallel for 3 times each time, and the measurement probe is cleaned before each measurement.

2.8 Data Statistics

Use EXCEL software to make descriptive statistics for each measured value, including quantity, mean value, minimum value and maximum value, etc.SPSS analysis software was used to test the significance of the difference normal distribution by Shapiro-Wilk Test.Sig.(Two-sided)> 0.05, normal distribution is presented, paired t-test is performed, and significant difference level α is 0.05.If Sig.If (two-sided)< 0.05, it is non-normal distribution, Wilcoxon signed rank test is performed, and the level of significant difference α is 0.05.

3. Results

3.1 Supermolecular HA Transdermal Experiment

The in vitro transdermal results of fluorescent-labeled supramolecular HA solution and non-supramolecular HA solution are shown in Fig 3.The results of fluorescence staining showed that the transdermal effect of HA decreased with the increase of its MW.At the same MW, the transdermal effect of supramolecular HA was much better than that of non-supramolecular HA, and it could penetrate deep skin.In the moderately polymerized state with a MW of 100- 1,800 kDa and in the highly polymerized state with a MW greater than 1,800 kDa, supramolecular HA can still penetrate deep into the skin, while non-supramolecular HA can hardly penetrate deep into the skin.

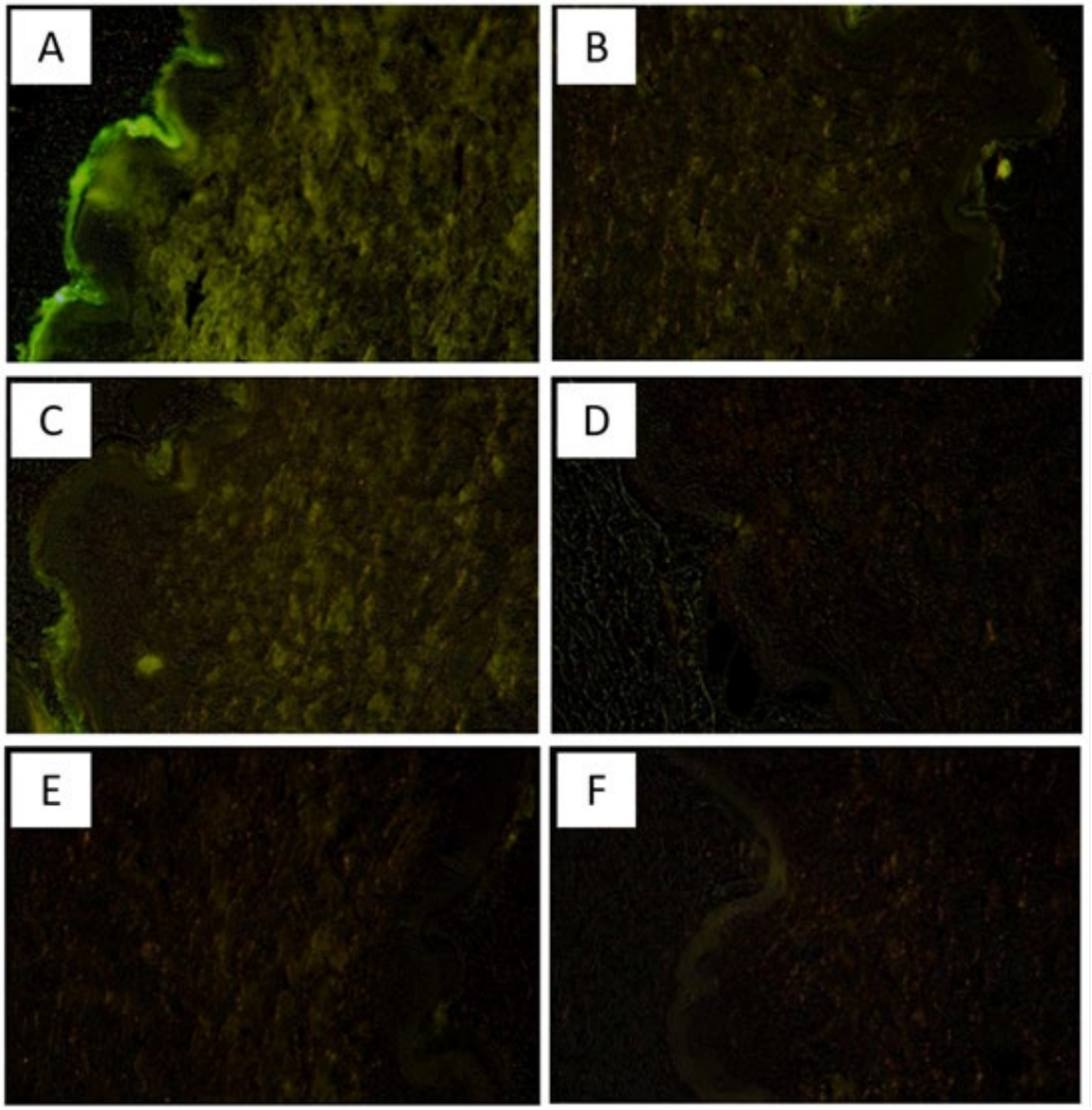

Figure 3: In vitro transdermal experiment of supramolecular HA and non-supramolecular HA with different MW.After 2h of transdermal reaction, the fluorescent-labeled HA (A\B: low molecular weight HA of 10-100 kDa, C\D: middle molecular weight HA of 100-1,800 kDa, E\F: high molecular weight HA of more than 1,800 kDa) were cut into paraffin sections and observed under confocal laser scanning microscope. A, C and E are the transdermal results of supramolecular HA, and B, D and F are the transdermal results of non-supramolecular HA.

3.2 Human Skin Moisturizing Experiment Volunteers Recruitment

A total of 24 valid subjects meeting the volunteer enrollment criteria were finally recruited, including 4 males and 20 females, aged from 20 to 63 years, with an average age of 50.50±2.66 years.Information of enrolled volunteers is provided in Table 1.

Table 1: Information of effective volunteers enrolled in human skin moisturizing experiment.

Number	Subject Name	Gender	Age
1	LZR	Female	60
2	ZWY	Female	59
3	CY	Female	44
4	SCX	Female	60
5	LG	Male	60
6	CZL	Female	41
7	HML	Female	62
8	HZY	Female	61
9	WHW	Female	57
10	ZFJ	Female	58
11	SZQ	Female	24
12	ZYP	Male	42
13	ZJ	Male	62
14	GJR	Female	54
15	CSH	Female	58
16	GJG	Female	58
17	ZDJ	Male	20
18	BCL	Female	63
19	DJH	Female	52
20	ZHY	Female	53
21	HRR	Female	53
22	ZLJ	Female	49
23	ZCQ	Female	41
24	WYJ0	Female	21

3.3 Moisturizing Experiment of Supramolecular HA on Human Skin

By measuring the skin capacitance value in the test area of volunteers, it was found that the water content of stratum corneum was significantly increased at 4h and 8 h after the application of supramolecular HA and non-supramolecular HA compared with the blank group (Figure 4).The water content of stratum corneum increased by 25.83% ($P<0.001$) and 17.21% ($P<0.001$) respectively at 4h and 8 h after the administration of supramolecular HA.The water content of stratum corneum increased by 12.66% ($P<0.001$) and 7.83% ($P<0.001$) at 4h and 8 h after application of non-supramolecular HA, and the increase of water content of stratum corneum of supramolecular HA was higher than that of non-supramolecular HA ($P<0.001$).

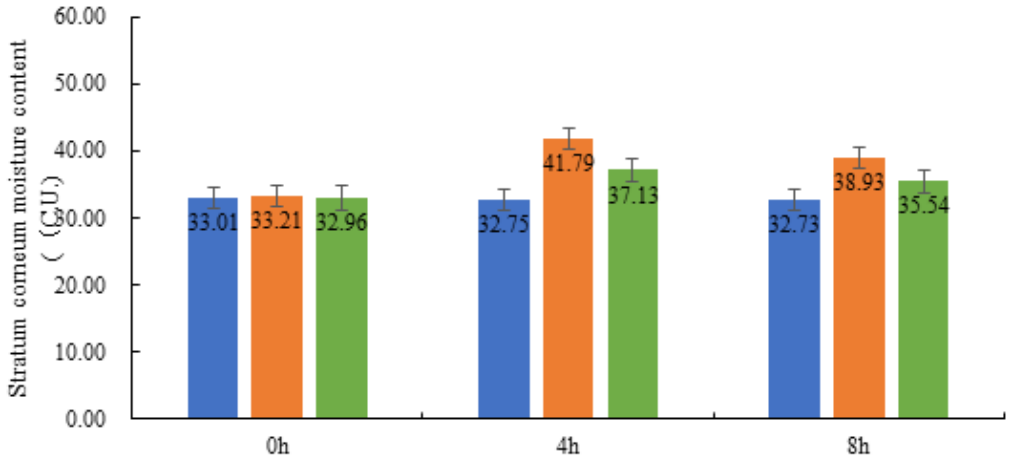

Figure 4: Change trend of moisture content in stratum corneum of volunteers 'skin in test area.The blue histogram represents the area coated with ultrapure water, the orange histogram represents the area coated with supramolecular HA, and the green histogram represents the area coated with non-supramolecular HA.

In order to compare the moisturizing effect of supramolecular HA and non-supramolecular HA, we calculated the increase in water content of the stratum corneum before application of HA and 4 and 8 hours after application.The results showed that the increase of skin stratum corneum water content of supramolecular HA was significantly higher than that of non-supramolecular HA at 4h and 8 h after application (Figure 5), and the increase of skin water content of supramolecular HA at 8 h after application was higher than that of non-supramolecular HA at 4h.

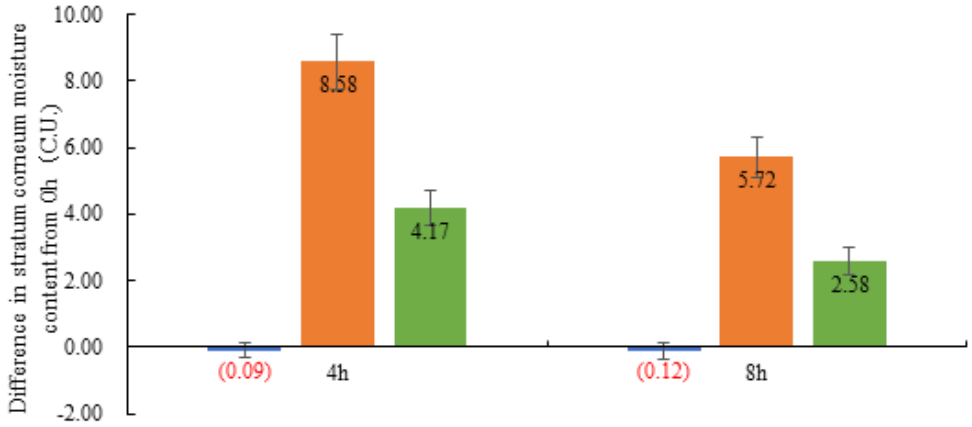

Figure 5: Increased moisture content of stratum corneum.The blue histogram represents the area coated with ultrapure water, the orange histogram represents the area coated with supramolecular HA, and the green histogram represents the area coated with non-supramolecular HA.

4. Discussion

HA is abundant in the dermis of the skin in humans, providing a spatial framework for the distribution of collagen fibers and elastin, forming together a skin scaffold to maintain skin tissue stability and skin elasticity.If one of three is missing, it can accelerate the f skin aging and formation of wrinkles.But studies have shown that human skin epidermal thickness shrinks by an average of 6.4% every 10 years.In the process of skin aging, the production of extracellular matrix (ECM) such as HA, collagen and elastin decreases, while the expression of matrix metalloproteinases (MMPs) increases, which increases the degradation of extracellular matrix and further accelerates the collapse of skin structure [14].Therefore, exogenous supplementation HA is an important approach to restore aging skin structure and maintain skin function.

As one of the components of human connective tissue and synovial fluid, HA is one of the world's most widely used skin fillers because of its high biocompatibility.Commonly used for periocular wrinkle like crows-feet and glabellum wrinkle, and nasolabium wrinkle [20,21].

However, The 500 Dalton rule for the skin penetration of chemical compounds and drugs, on the one hand, provides protection

for the body, but on the other hand, it also sets up obstacles for functional compounds such as macromolecular drugs and cosmetics to enter the skin.

As a polysaccharide, the MW of HA is positively correlated with the number of water bound by HA and negatively correlated with the depth of penetration into the skin.Essendoubi et al. 2016 showed that hyaluronic acid of different MWs can be absorbed by the skin, with HMW (1,000 kDa-1,400 kDa) HA mainly penetrating into the stratum corneum of the skin, while LMW (20kDa-300kDa) HA penetrates deeper [22].

Thus, microneedle injection has become the most widespread means of dermal exogenous HA supplementation.However, considering the invasiveness of injection, this method has a series of disadvantages such as infection risk, long recovery period, specific operators and locations, and high cost.Therefore, it is of great significance to find a non-invasive method that can make HMW HA penetrate deep into the skin

Brown et al. (1999) demonstrated that HA is not only absorbed by the skin in a passive diffusion manner, but also has an active transport absorption capacity [23].This suggests that HMW HA has the potential to penetrate deep into the skin, but its physicochemical properties limit this possibility.

Previous studies have also used liposomes to encapsulate HMW HA molecules and deliver them deep into the skin by modifying their lipophilicity [24]. However, this method has a series of disadvantages such as complicated preparation process, high cost, storage limitation, etc., and cannot be widely applied on a large scale.

In this study, the common HA molecule was transformed into supramolecular state by using ionic liquid induction, and then the skin penetration mode of HA molecule, especially HMW HA molecule, was changed, so that it could penetrate the stratum corneum and enter deeper skin non-invasively.It can be seen from the experimental results that the transdermal ability of HA with HMW is obviously improved after being activated by ionic liquid to present supramolecular state, and its ability to maintian water molecules and replenish water to skin is also greatly improved.

The preparation process of supramolecular HA in this study is relatively simple, low cost, simple storage conditions, and convenient for subsequent industrial large-scale production.

As for the comparison of the transdermal ability of supramolecular HA with different MW, it still accords with the rule that the lower the molecular weight, the better the transdermal effect. However, the HMW HA that remain outside the stratum corneum of the skin form a protective barrier on the skin surface, preventing water loss from the skin.We speculate that if supramolecular HA with different MW is made into a certain proportion of mixed solution, using the strategy,which is MMW and LMW HA transdermal hydration and repair skin tissue,HMW HA to create a protective barrier its moisturizing effect will be better than using a single certain MW supramolecular one. Therefore, the following experiments can compare the hydration effect of mixed supramolecular HA with that of pure HMW,MMW and LMWsupramolecular HA to verify the effectiveness of the above strategy.At the same time, the optimal mixing ratio of supramolecular HA with different MW in the mixed supramolecular HA solution can be explored.

In addition, the results of this study confirmed that HMW HA can penetrate deep skin, suggesting that supramolecular HA has unique physical and chemical characteristic.This also provides ideas and references for the development and improvement of other transdermal functional compounds in supramolecular state.

Contributions

P.H. led the study. P.H., H.W. and C.L. conceived the study and designed all analyses. H.W., Z.J. and X.S. accomplished HA molecular fluorescence labeling experiment. H.W. and T.X. prepared supramolecular hyaluronic acid. H.W. and X.S. accomplished the transdermal penetration of supramolecular HA experiment in vitro. X.L. and L.W. recruited human skin moisturizing experiment volunteers. H.W. and S.W. accomplished moisturizing experiment of supramolecular HA on human skin. H.W., Z.J., X.L. and L.W. conducted the uncertainty analysis. P.H., C.L., H.W., and Z.J. interpreted the final results. H.W., Z.J., H.W. and C.L. wrote the paper. P.H., Z.J. and H.W. produced the graphical representation of the results. All authors contributed to revising the paper.

Ethics Declarations

Ethical Approval

All of the experimental procedures involving human studies were conducted in accordance with the Ethics Commitee Of Chinese PLA General Hospital, Beijing ,China and was approved by the Ethics Commitee Of Chinese PLA General Hospital, Beijing ,China.

Statement of Animal Ethics

This study was approved by the Administration Committee of Experimental Animals, Beijing ,China.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

The original contributions presented in this study are included in the article.

Acknowledgement

Huaxi Biotechnology Co. Ltd. has provided all different MW of HA and support to the HA quality inspection. We are grateful to Pan Wang (Peking University Third Hospital, Beijing, China) for the experiment volunteers recruitment work and the valuable advice for the volunteers experiment grouping. Weibin Wang - School of Basic Medical Sciences, Peking University, Beijing, China) made a great contribution to the data analyses and provided the paper guidance.

5. Conclusion

In this study, we prepared HA with different MW by ion-induced method, and confirmed that HA with different MWcan penetrate into deep skin.Compared with ordinary HA, supramolecular HA can increase the skin moisture content by more than 2 times, and can help the skin lock moisture for a longer time and delay the skin water loss process.

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